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TITLE: AGE-RELATED DNA METHYLATION CHANGES AND NEOPLASTIC TRANSFORMATION OF THE HUMAN PROSTATE

PRINCIPAL INVESTIGATOR: BERNARD KWABI-ADDO Ph.D.

CONTRACTING ORGANIZATION: HOWARD UNIVERSITY
WASHINGTON, DC 20060

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BERNARD KWABI-ADDO, Ph.D.

Email: bkwabi-addo@howard.edu

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**14. ABSTRACT** **PURPOSE:** There is abundant evidence to suggest that DNA methylation changes may appear earlier during prostate cancer development than genetic changes, as well as more commonly and consistently. The purpose of the present study is to investigate whether aberrant methylation in normal prostate tissues may in itself be a pathologic event that increases with age. **RESULTS:** Using methylated CpG island amplification coupled with CpG promoter microarray, I have identified several novel genes that are differentially methylated in the human prostate. Quantitative methylation analysis using pyrosequencing technique shows hypermethylation of these genes in prostate cancer tissues compared with matched benign prostate tissues from the same patients. Furthermore, I observed methylation changes as a function of age for several genes and that the methylation profiles were different between samples from African American compared to Caucasian men. **CONCLUSION:** I have identified several novel genes as potential (ethnic sensitive) biomarkers for prostate cancer detection.

**15. SUBJECT TERMS**
Epigenetic DNA methylation changes, microarray analysis, pyrosequencing, RT-PCR, prostate cancer

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## Introduction

Prostate cancer is still a common malignancy and a leading cause of cancer death among men in the United States. The progressive acquisition of genomic alterations is a defining feature of all human cancers, including prostate cancer. Prostate cancer cells are known to carry a variety of genetic defects, including gene mutations, deletions, translocations, and amplifications, that endow the cells with new capabilities for dysregulated proliferation, inappropriate survival, tissue invasion and destruction, immune system evasion, and metastasis.[1] More recently, it has become apparent that prostate cancer cells also carry epigenetic defects, including changes in cytosine methylation patterns and in chromatin structure and organization, which are equivalent to genetic changes in effecting and maintaining neoplastic and malignant phenotypes.[2] For human prostate cancer, abundant evidence has accumulated to suggest that somatic epigenetic alterations may appear earlier during cancer development than genetic changes, as well as more commonly and consistently. Furthermore, epigenetic changes tend to arise in association with age[3] and/or in response to chronic or recurrent inflammation leading to cell and tissue damage.[4]

Epigenetic inactivation of genes in cancer cells is largely based on transcriptional silencing by aberrant CpG methylation of CpG-rich promoter regions.[5, 6] Aberrant promoter methylation of GSTP1, encoding the π-class glutathione *S*-transferase (GST), an enzyme capable of detoxifying electrophilic and oxidant carcinogens remains the most common somatic genome abnormality of any kind (>90% of cases) reported thus far for prostate cancer, appearing earlier and more frequently than other gene defects that arise

during prostate cancer development.[7] Since the recognition that the *GSTP1* CpG was frequently hypermethylated in prostate cancer, more than 40 genes have been reported to be targets of DNA hypermethylation-associated epigenetic gene silencing in prostate cancer cells.[8] Despite the increasing number of aberrantly methylated genes in prostate cancer, only a few genes show promise as prostate cancer biomarkers for early diagnosis and disease risk assessment.

In this study, I sought to investigate whether aberrant methylation in the normal prostate tissues may in itself be a pathologic event that increases with aging. By analyzing methylation on a genome wide scale using methylated CpG island amplification (MCA) coupled with CpG promoter microarray[9] in prostate cancer LNCaP cell line I have identified several novel methylated genes in human prostate cell line. Identification of age-related methylated genes in the normal prostate has important implications for the study of DNA methylation in prostate cancer etiology and for the development of biomarkers for the detection of this disease.

**BODY**

As outlined in my Statement of work, I seek to accomplish 3 main tasks during the 3 years of funding. I have made substantial progress in 2 out of these 3 tasks. A manuscript describing "differential DNA methylation profiles reveal novel pathways in prostate carcinogenesis" is in preparation for submission to the Journal of Clinical Cancer Research and an abstract from this work was presented at the American Association for Cancer Research 2009, Annual Meeting in Denver, CO. A copy of this abstract is attached below.

**Specific Aim 1. Comprehensive age-dependent DNA methylation analysis of novel genes in the human prostate**

Several genes have been shown to be hypermethylated in prostate cancer. [10] However, hypermethylation of genes in normal prostate tissues may be an early event predisposing cells to neoplastic transformation. I have recently shown that some genes which are hypermethylated in prostate cancer tissue may undergo methylation in normal prostate tissues in an age-dependent manner. To my knowledge, this was the first study to directly examine the relationship between methylation and age in human prostate tissues. This study clearly demonstrated that methylation starts in normal prostate tissues as a function of age and markedly increases in cancer.[3]

A global profile of genes that are methylated in normal prostate tissues as a function of age would serve to identify candidate genes that are hypermethylated as an early event in the transition from normal prostate cells to prostate cancer and ultimately provide insight into understanding the molecular mechanisms underlying DNA methylation, aging and neoplastic transformation. In this aim, I proposed to carry out a comprehensive analysis of novel methylated genes that I have identified using a combination of methylated CpG island amplification coupled with CpG promoter microarray to ascertain if methylation status can provide reliable information for the detection of prostate cancer.

*Detection of Methylated CpG Island Using MCA-CpG promoter microarray*

From my preliminary studies, I have used methylated CpG island amplification (MCA) technique[11] coupled with CpG promoter microarray to identify several novel methylated

genes in the human prostate cancer line LNCaP. I identified more than 300 differentially hypermethylated loci of which approximately 50 were unique promoter associated CpG islands. Similarly, I identified about 374 differentially hypomethylated loci in the LNCaP cells. Interestingly, 349 of these hypomethylated probes mapped to repetitive elements and only 25 loci were unique promoter associated CpG islands as revealed by BLAST (www.ncbi.nlm.nih.gov) and BLAT (genome.ucsc.edu) searches. Several of these genes have previously been reported. Novel methylated genes of particular interest in the current study included PAX9, RPRM, CDH11, SPARC, FOXN4, TIMP3, and TCF3. These genes were chosen for initial studies based on either their chromosomal localization, their regulatory function or whether they may be important in prostate cancer etiology as shown in Table 1.

| No. | Gene name | Bidirectional | Locus | Location | Previously reported |
|---|---|---|---|---|---|
| 1. | PAX9 | No | 14q13.3 | 36202075-36202382 | Yes |
| 2. | RPRM | No | 2q23.3 | 154042696-154044004 | Yes |
| 3. | CDH11 | No | 16q22.1 | 63711958-63715365 | No |
| 4. | SPARC | No | 5q31.3 | 151046061-151047060 | Yes |
| 5. | FOXN4 | No | 12q24.11 | 108231010-108232761 | Yes |
| 6. | TIMP3 | No | 22q12.3 | 31527381-31528267 | Yes |
| 7. | TCF3 | No | 19p13.3 | 1596918-1598213 | No |

**Table 1** shows a list of genes identified as differentially methylated in prostate cancer cell line; LNCaP.

*DNA methylation analysis in cell lines.*

In order to validate the MCA-microarray results, I investigated the methylation status of 10 genes differentially methylated (8 predicted hypermethylated and 2 predicted unmethylated) in a panel of 21 cell lines. To accomplish this, high molecular weight

DNA was extrac ted from the cell lines and m odified using sodium bisulfite treatm ent. Bisulfite P CR pri mers were designed base d on bisulfite/converted sequence from the CpG ensuring that the bisulfite-PCR pri mers avoid CpG si tes and that they are designed as close to the trans cription start site as possi ble. The bisulfite prim ers were then used in a PCR reaction with the bisulfite treated ge nomic DNA from the cell lines. A two step nested PCR reaction was carried o ut using 2 se ts of different PCR prim ers. This helps improve the specificity and purity of the PCR products used in the pyrosequencing reaction. O ne of the prim ers (reverse prim er) in the 2 $^{nd}$ s tep PC R reac tion was biotinylated in order to cr eate a single-stranded DNA te mplate for the pyrosequencing reaction. T he PCR products were imm obilized on streptav idin-sepharose b eads (Amersham), washed, denatured, and the biotinyl ated strands releas ed into an annealing buffer containing the sequencing prim er. Pyrosequencing was perform ed using the PSQ HS96 Gold SNP Reagents on a PSQ 96 HS machine (Biotage).

I have carried out the methylation analysis in a 21-cell line panel (Fig 1). All of the genes investigated showed methylation in at least a s et of cell lin es from one cancer site. E ight of the 10 analyzed gene s showed hyperm ethylation in at least one of the thr ee prostate cancer cell lines. The PAX9 and CYP27B1 ge nes were unm ethylated in prostate cancer cell lines. Individual cell line s showed a range of m ethylation frequency. The RPRM, SPARC, NKX2-5, RASSF1A gene s showed hyperm ethylation in the prostate cancer cell lines. The TIMP3, RPRM and RASSF1 A al so showed hyperm ethylation in the immortalized prosta tic prim ary epithelial cells (pNT1A ce lls). I t is like ly tha t the methylation observed in pNT1A c ells coul d be derived from repeated passages and selection during cell culture. The CDH 11, FOXN4and CYP27B1 genes did not display

hypermethylation in the prostate cell lines. However, individual prostate cancer cell lines showed varied methylation frequency as was observed in the cancer cell lines from different tissue sites.

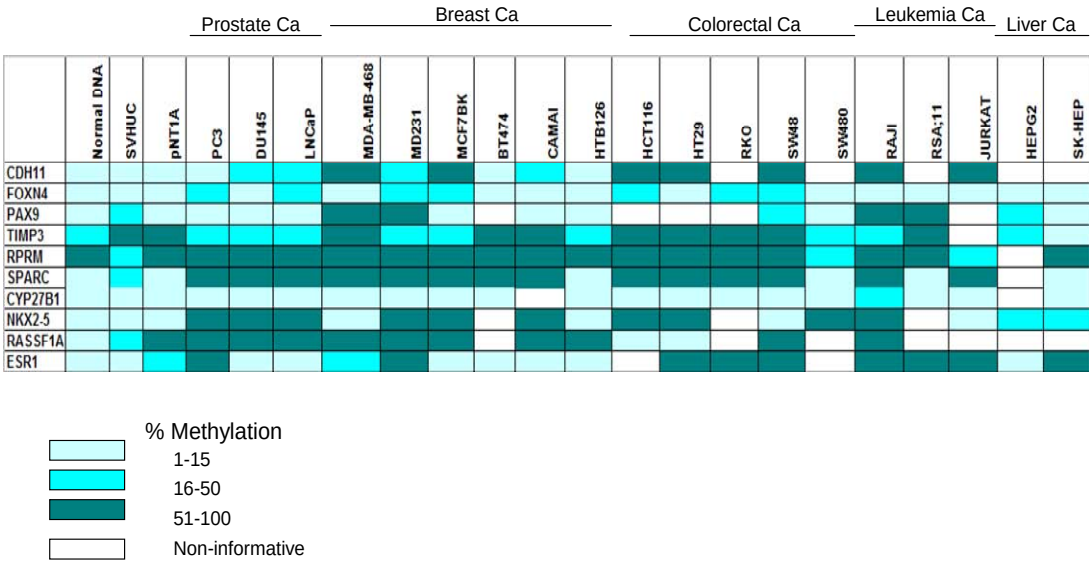


**Fig 1.** The methylation status of 10 genes was investigated in a 21-cell line panel by pyrosequencing. Cell lines include a tumorigenic urothelial epithelium SVHUC cell line and the primary prostatic epithelial cell line, pNT1a, both immortalized by SV40 transfection. The scale refers to the degree of methylation as measured by pyrosequencing

*DNA methylation analysis in prostate tissues*

Having established differential methylation of these genes in the panel of 21-cell lines, I next wanted to compare the level of methylation in normal and prostate cancer tissues. To accomplish this, I obtained matched pairs of benign and prostate cancer tissue samples from patients who have undergone radical prostatectomy (25 samples). High molecular weight genomic DNA was extracted from the tissue samples. Genomic DNA samples were modified using sodium bisulfite treatment and modified DNA used in pyrosequencing analysis as described above. Results presented in Fig 2 demonstrate that compared with the methylation data from normal prostate tissues, there is significantly

higher methylation in prostate cancer tissues for FOXN4, TIMP3, RPRM, SPARC and CYP27B1. On the other hand, CDH11 and PAX9 genes do not show a significant difference in DNA methylation between the normal and matched prostate cancer tissues.

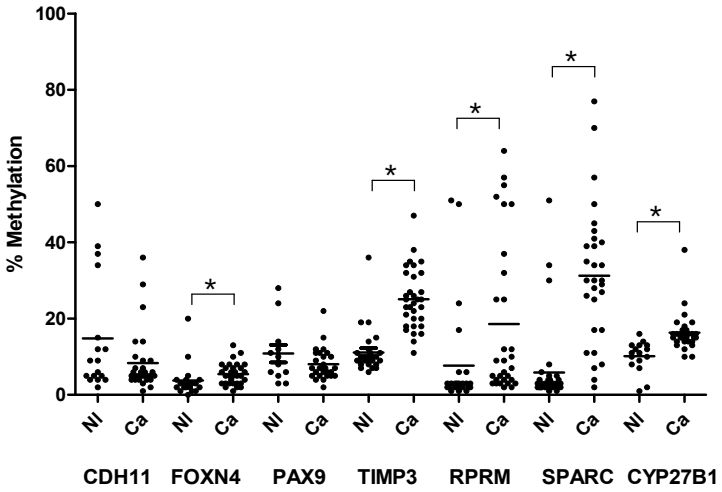


**Fig 2.** The % methylation level of novel genes in matched normal (Nl) versus prostate cancer (Ca) tissue samples from individual patients that had undergone radical prostatectomy. * shows statistically significant data as determined by Mann Whitney t-test, with significance set at the level of $p < 0.05$.

**Methylation and gene expression**

To investigate if methylation leads to gene silencing, I performed expression analysis using total RNA extracted from matched normal and prostate cancer tissue samples by quantitative RT-PCR for 6 genes, CDH11, PAX9, TIMP3, SPARC, FOXN4 and RPRM based on their potential function in prostate carcinogenesis as well as their frequency of methylation in cell lines and prostate tissues (Fig 3). I observed a good correlation between methylation frequency and expression to indicate that methylation leads to gene silencing. My ongoing study is to investigate whether the expression of those genes that were hypermethylated in our studies can be restored after treatment with the demethylating agent, 5-azadC and the histone deacetylase inhibitor.

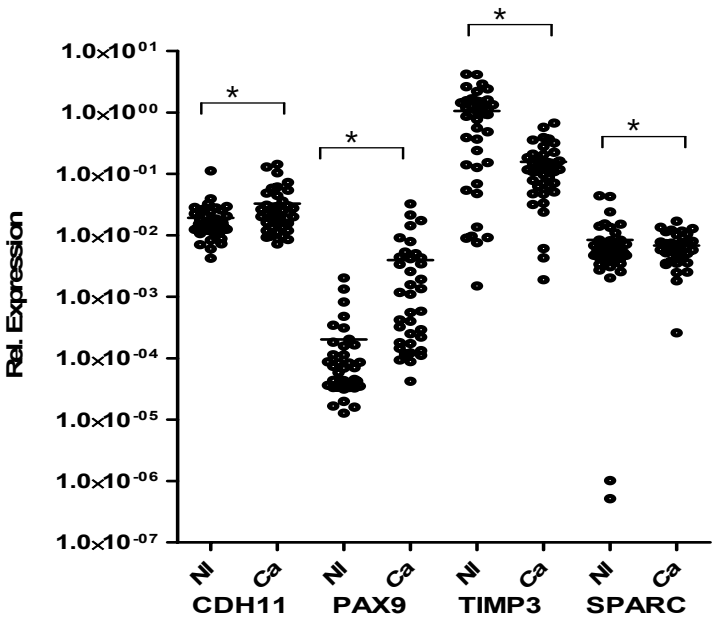


**Fig 3.** Relative expression of genes in matched normal (Nl) versus prostate cancer (Ca) tissue samples as determined by RT-PCR. * indicates statistically significant data.

**Specific Aims 1 & 2. Comprehensive age-dependent DNA methylation analysis and the association of DNA methylation levels and genetic predisposition**

There is documented evidence to demonstrate that gene inactivation by DNA methylation may play a role in the pathogenesis of prostate cancer. But the incidence and mortality of prostate cancer is approximately 1.6-fold higher among African-Americans as compared to Caucasians.[12] When compared with their white counterparts, black men have a greater tendency to present at a younger age and later stage of disease. [13] This clearly indicates that genetic factors and/or environmental exposure influences contribute to prostate cancer risks. For example, prostate cancer epidemiology supports the idea that dietary factors, especially fat may increase the risk of prostate cancer. [14] By investigating age-dependent methylation of several novel genes, it may be possible to identify a pattern for differential genes methylation in normal and prostate cancer tissues from different ethnic groups. This may help to explain the increased incidence and severity of prostate cancer

in African American men when compared to other ethnic groups. In addition, this may serve as a sensitive biomarker in one ethnic group when compared to the other.

In order to investigate if there is age-dependent DNA methylation changes in different ethnic groups, I studied DNA methylation changes of 8 genes; SPARC, RARb2, AR, TIMP3, GSTP1, NKX2.5, RASSF1A and CYP27B1 in DNA samples from African American (AA) and Caucasian (Cau) men as a function of age. For this study, I used normal prostate tissue samples from AA and Cau (between 25 – 80 years old) to further understand if one ethnic group has more propensities to methylation compared to the other group (Fig 4). I observed a significantly higher methylation in the samples from AA for RARb2, AR, NKX2.5 and RASSF1A. The higher methylation observed for these in AA appeared to increase with age except for AR gene where I did not see this effect. On the other hand, I did not see any significant differences in the methylation pattern for SPARC, TIMP3, GSTP1, and CYP27B1.

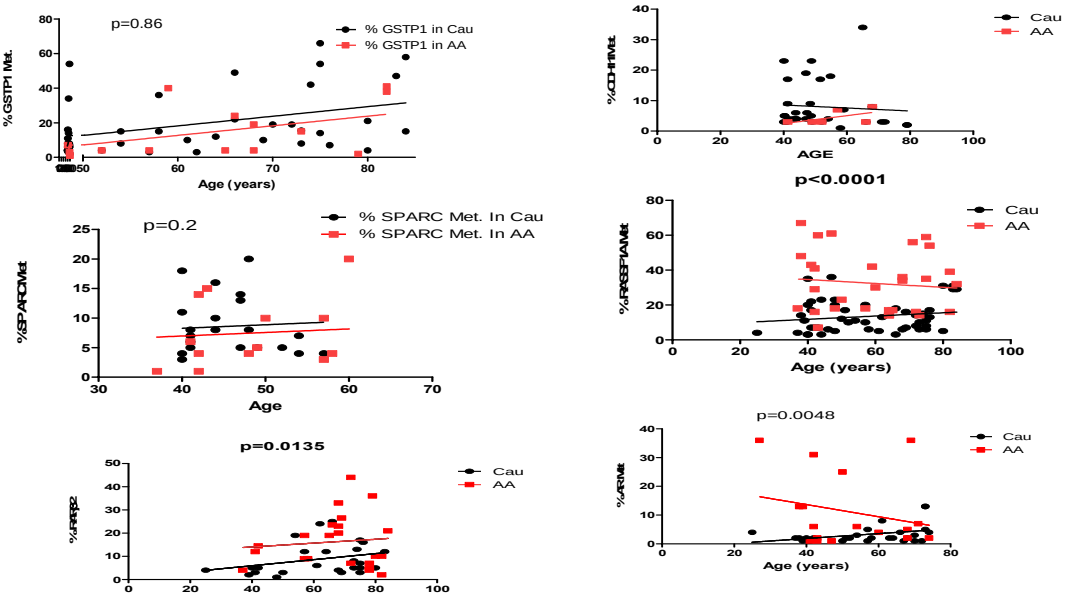

**Fig 4.** Age-related methylation analysis in normal prostate tissues. CpG islands for GSTP1, CDH11, SPARC, RASSF1A, RARβ2 and AR in 45-bisulfite modified genomic DNA extracted from normal prostate tissues (age range 25-80 years old) from both African American (AA) and Caucasian (Cau) men. Y-axis represents the percentage of methylated cytosines in the samples as obtained from pyrosequencing. Each CpG island has a different scale range. X-axis represents age in years.

## KEY RESEARCH ACCOMPLISHMENTS

- I have identified several novel methylated genes using methylated CpG island amplification (MCA) technique coupled with CpG promoter microarray in the human prostate cancer line LNCaP.

- I have shown differential methylation of these genes in a panel of 21-cell lines derived from prostate, breast, colorectal, leukemia and liver tissues using pyrosequencing as a quantitative approach to measure methylation status. In addition, I have demonstrated in prostate tissue samples that compared with the methylation data from normal prostate tissues, there is significantly higher methylation in prostate cancer tissues for FOXN4, TIMP3, RPRM, SPARC and CYP27B1. On the other hand, CHD11 and PAX9 genes do not show a significant difference in DNA methylation between the normal and matched prostate cancer tissues.

- I have carried out comprehensive age-dependent DNA methylation analysis of these genes in normal prostate tissues derived from African American and Caucasian men organ donor or autopsy samples. Results indicate a significantly higher methylation in the samples from AA for RARβ2, AR, NKX2.5 and RASSF1A. The higher methylation observed in the AA men samples appeared to

increase with age except for AR gene wher e I did not see this effect. On the other hand, I did not see any significant differe nces in the m ethylation pattern for SPARC, TIMP3, GSTP1, and CYP27B1.

## REPORTABLE OUTCOMES

- **AACR Annual Meeting- Differential DNA methylation profiles reveals novel pathways in prostate carcinogenesis. (2009) Denver CO.**
- **Manuscript in preparation for submission to the Journal of Clinical Cancer Research**

Conclusion

I have used m ethylated CpG island am plification (MCA) technique coupled with CpG promoter microarray in the prostate cancer ce ll line, LNCaP to iden tify novel methylated genes. Using this approach, we have identified 50 unique promoter associated CpG island to be d ifferentially hyp ermethylated in th e L NCaP cell line and 25 unique promoter associated CpG island s to b e dif ferentially h ypomethylated in the L NCaP cell line . Several of these differentially m ethylated genes have been previously reported. Novel methylated genes of particular interest in our current study included PAX9, RPRM, CDH11, SPARC, FOXN4, and TIMP3. These genes were chosen for initial studies based on either their chromosomal localization, their regulatory function and therefore represent a subset of genes where silenc ing may play a role in the prostate can cer etiology and/or progression.

I have used pyrosequencing to quantitatively measure the methylation levels of these genes in a panel of cell lines as well as in prostate tissues. My results demonstrate differential methylation of these genes in the cell lines. Methylation analysis of these genes in human prostate tissues showed significantly higher methylation in the prostate cancer tissues in comparison to the normal prostate tissues for FOXN4, TIMP3, RPRM, SPARC and CYP27B1. On the other hand CHD11 and PAX9 genes did not show a significant difference in DNA methylation between the normal and matched prostate cancer tissues. While I have yet to definitively demonstrate that methylation leads to gene silencing, the observation of an inverse association between DNA methylation and gene expression as determined by RT-PCR analysis suggests that DNA methylation leads to gene silencing.

The FOXN4 gene belongs to the human forkhead-box (FOX) gene family and de-regulation of the FOX genes has been suggested in several diseases including congenital disorders, diabetes mellitus, or carcinogenesis.[15] However, there are no reports of epigenetic changes of FOXN4 gene in prostate cancer. The tissue inhibitor of metalloproteinase 3 (TIMP3) is believed to play a significant role in controlling extracellular matrix remodeling and has been previously shown to be methylated in urine sediment of prostate cancer patients. Nonetheless, TIMP3 persistently shows low frequency of methylation in normal prostate tissues which may limit it's' usefulness as diagnostic marker in urine DNA. [16] Reprimo (RPRM) is a putative mediator of p53-mediated cell cycle arrest at the G2 phase of the cell cycle. Aberrant methylation of RPRM has been reported in a number of cancer types including prostate cancer. [17] The

secreted pro tein ac idic and rich in cyste ine (S PARC) is reported ly d etrimental to the growth of ovarian cancer cells and has been reported to be hyperm ethylated in several cancers including prostate cancer.[18] Previous reports has shown that the 25-Vitamin –D3-1alpha –hydrolase (CYP27B1) is downregulat ed during prostate tu mor pathogenesis and that treatm ent of prostate cells with the methylation inhibitor 5-aza-2'-deoxycytidine together with the dea cetylation inhibito r trichosta tin A resu lted in eleva tion of CYP27B1.[19] However, in m ost of these studies, the m ethylation status of these genes have been identified by either indirect observation or thr ough re-activation by pharmacological drug treatm ent. In m y pres ent study, I have used quantitative DNA methylation analysis to measure differences in the methylation level between norm al and prostate cancer tissues and I want to correlate the methylation pattern of these genes with clinical and pathological data to as certain the usefulness of the ge ne(s) as diagnostic marker(s) for prostate disease detection.

I have compared the methylation profiles of these genes in normal prostate tissue samples from African American and Caucasian men. My analysis indicates significant differences in the m ethylation patterns for these 2 ethn ic groups. Overall, I observed significantly higher methylation as a function of age for the samples obtained from African Am erican when com pared to Caucasian m en. The high er m ethylation observed suggests that if methylation is lead ing to silen cing of key regulato ry g enes in the prosta te c ancer pathogenesis, this event could contribute to higher incidence of prostate cancer observed in Af rican Am erican m ales. Inter estingly, I did not detect higher methylation f or the GSTP1 gene in our studies. However, ot her studies have reported that GSTP1 hypermethylation to be a very sensitive di agnostic m arker for African Am erican m en

compared to other ethnic group. [20] In this particular report, they analyzed a region of the GSTP1 CpG island which is different from the CpG island that I investigated. It is possible that their site is more prone to higher methylation in the African American samples. I am currently investigating the methylation status at other sites of the GSTP1 CpG island.

Overall, I have identified several novel genes as potential biomarkers for prostate cancer disease detection and some of these genes could serve as ethnic sensitive biomarkers for prostate cancer.

**FUTURE WORK WILL FOCUS ON**

1. In-depth methylation analysis of genes described above. Some of my pyrosequencing assay designs for methylation analysis do not identify all methylation sensitive regions. For some genes that do not appear to show methylation as a function of age in my samples, it may necessary to study other sites of the particular gene CpG islands. In addition I will carry out comprehensive DNA methylaiton analysis of additional novel genes which I have identified using methylated CpG island amplification (MCA) coupled with DNA microarray of human CpG island sequences.
2. I will correlate the methylation level of these genes with clinical and pathological data for prostate tissue samples from individual patients. The objective is to identify novel genes that would be potentially useful as diagnostic biomarkers for prostate disease detection.

3. I will continue to investigate the methylation pattern of additional genes as a function of age for samples obtained from African American and Caucasian men in order to identify methylated genes that could be potentially useful as biomarkers for one ethnic group compared to the other.
4. One of the genetic mechanism that may help in explaining why some men with prostatic intraepithelial neoplasia (PIN) never develop invasive prostate cancer, whereas other men may develop invasive prostate cancer could be due to differences in the frequency of methylated genes in these two groups of men with prostate disease. By comparing gene methylation profiles in these 2 groups of men it may be possible to identify genes which demonstrate higher methylation frequency in one group versus the other. Furthermore, it is known that epigenetic DNA methylation changes occurs very early in the pathophysiological of prostate disease and this may lead to other somatic mutations in the later stages in the disease pathway. I will study the mutational status of several methylated genes with key regulatory functions, it may be possible to identify novel genes that are methylated and/or mutated at higher frequency in one group of men versus the other group. This would contribute to further understanding the molecular mechanisms underlying the progression from PIN to invasive prostate cancer.
5. Finally, for novel gene(s) that appears to be a potential candidate as a diagnostic biomarker, I will study the biological function of such gene(s) in vitro prostate cancer cell lines to ascertain their role in neoplastic transformation of the human prostate. This would be accomplished by over-expressing recombinant vectors encoding for the gene product and assaying the effects on cell proliferation and/or

apoptosis. Alternately siRNA that targets the gene would be used to knock-down the gene expression to evaluate its effect on prostate cancer cell growth.

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21. *Int J Cancer* 2005 August 20;116(2):174-81.

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**Session Title:** DNA Methylation 2

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**Location:** Hall B-F, Poster Section 19

**Poster Section:** 19

**Poster Board Number:** 20

**Author Block:** *Bernard Kwabi-Addo*, *Songping Wang*, *Jaroslov Jelinek*, *Jean Pierre Issa*, *Michael Ittmann*. Howard Univ. Cancer Ctr., Washington, DC, MD Anderson Cancer, Houston, TX, Baylor College of Medicine, Houston, TX

The progressive acquisition of genomic alterations and epigenetic defects including changes in cytosine methylation patterns is a defining feature of all human cancers including prostate cancer. For human prostate cancer, abundant evidence has accumulated to suggest that somatic epigenetic alterations may appear earlier during cancer development as well as more commonly and consistently.

To identify novel CpG islands that are aberrantly methylated in prostate cancer, we used methylated CpG island amplification (MCA) couple with promoter CpG microarray analysis in the prostate cancer cell line, LNCaP. We identified more than 300 differentially hypermethylated loci of which approximately 50 were unique promoter associated CpG islands. We identified 21 clones to correspond to hypermethylated genes that maybe important in prostate carcinogenesis including Pax9, Reprimo, Cadherin 11, Notch1, Osteonection, netrin 4, Foxn4, Timp3, and Flt1. Similarly, we identified about 374 differentially hypomethylated loci in the LNCaP cells. However, 349 of these probes mapped to repetitive elements and only 25 loci were unique promoter associated CpG islands including the transcription factors TCF3 and ZNF306, Cadherin 12 and PTPRN2. The gene expression patterns of these genes were verified in prostate cell lines and tissue samples. Hypermethylation resulted in down-regulation of several genes including Osteonectin and Cadherin 11 in the prostate cancer cell lines and tissues in comparison to normal prostatic primary cells and benign prostate tissues. Hypomethylation on the other hand, led to the activation of several genes including Cadherin 12 and TCF3.

These data suggests epigenetic modulation of several genes and transcription factors in prostate cancer. Cumulative effect of these epigenetic changes may not only ensure cancer of the prostate but may also contribute to the metastasis of prostate cancer cells to other tissue sites.

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